THE GENE ENCODING IMIDAZOLE ACETOL PHOSPHATE AMINOTRANSFERASE IN ZYMOMONAS MOBILIS: A MEMBER OF A COMPLEX OPERON?

BY

WEI GU

A THESIS PRESENTED TO THE GRADUATE SCHOOL
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Wei Gu

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Chairman: Roy A. Jensen
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The gene encoding imidazole acetol phosphate aminotransferase in Zymomonas mobilis, designated as hisH, is located immediately upstream of tyrC, encoding cyclohexadienyl dehydrogenase. A plasmid containing hisH was able to functionally complement an Escherichia coli auxotroph which lacked the homologous aminotransferase. DNA sequencing of hisH revealed an open reading frame of 1110 bp, encoding a protein of 40,631 Da. A typical sigma-70 type of promoter sequence was identified in the upstream flanking region of hisH. The codon usage of hisH exhibited a similarity with Z. mobilis genes encoding low-abundance proteins. A comparison of the amino acid sequence of hisH with those of other known imidazole acetol phosphate aminotransferases showed distinct homology. Several conserved regions having known functional roles for aspartate aminotransferases were evident in multiple

alignment. The cloned hisH product was purified from E. coli, and estimated by SDS-PAGE to have a molecular mass of 40,000 Da. The native enzyme had a molecular mass of 85,000 Da as determined by gel filtration, which suggested that the native enzyme consisted of two identical subunits. The purified enzyme also possessed aromatic amino acid aminotransferase activities. It was concluded that a single protein having broad substrate specificity exists based upon the constant ratio of activities obtained with different substrates following a variety of physical treatments. The purified enzyme did not require addition of pyridoxal-5'-phosphate, but dependence upon this cofactor was demonstrated following resolution of the enzyme and cofactor by hydroxylamine treatment. Kinetic data showed the classic ping-pong mechanism expected for aminotransferases. K_m values of 0.17 mM, 3.39 mM, and 43.48 mM for histidinol phosphate, tyrosine, and phenylalanine were obtained. The gene structure around hisH-tyrC suggested an operon organization. Although the hisH-tyrC cluster is reminiscent of the hisH-tyrA component of a complex operon in Bacillus, the linked tryptophan-pathway genes found in Bacillus were not clustered with hisH-tyrA in Z. mobilis.

INTRODUCTION

Imidazole acetol phosphate (IAP) aminotransferase catalyzes the formation of histidinol phosphate by transamination of imidazole acetol phosphate in histidine biosynthesis (Fig. 1). Although a number of aminotransferases are not essential for growth because of the enzymatic backup attributed to the overlapping specificities of the intracellular repertoire of aminotransferases (Jensen and Calhoun, 1981), IAP aminotransferase is essential in all organisms studied. Thus, mutants which lack IAP aminotransferase activity are auxotrophic for histidine in Escherichia coli (Garrick-Silversmith and Hartman, 1970), Bacillus subtilis (Weigent and Nester, 1976a), Corynebacterium glutamicum (Araki and Nakayama, 1970), Halobacterium volcanii (Conover and Doolittle, 1990), and Streptomyces coelicolor (Limauro et al., 1990).

Genes involved in histidine biosynthesis form a single operon in Salmonella typhimurium (Ames and Garry, 1959; Sanderson and Roth, 1988) and E. coli (Grisolia et al., 1983; Bachmann, 1990). In B. subtilis all genes of histidine biosynthesis also map together, except for hisH (Piggot and Hoch, 1985). It is interesting that hisH, the gene encoding IAP aminotransferase in B. subtilis, is linked with the

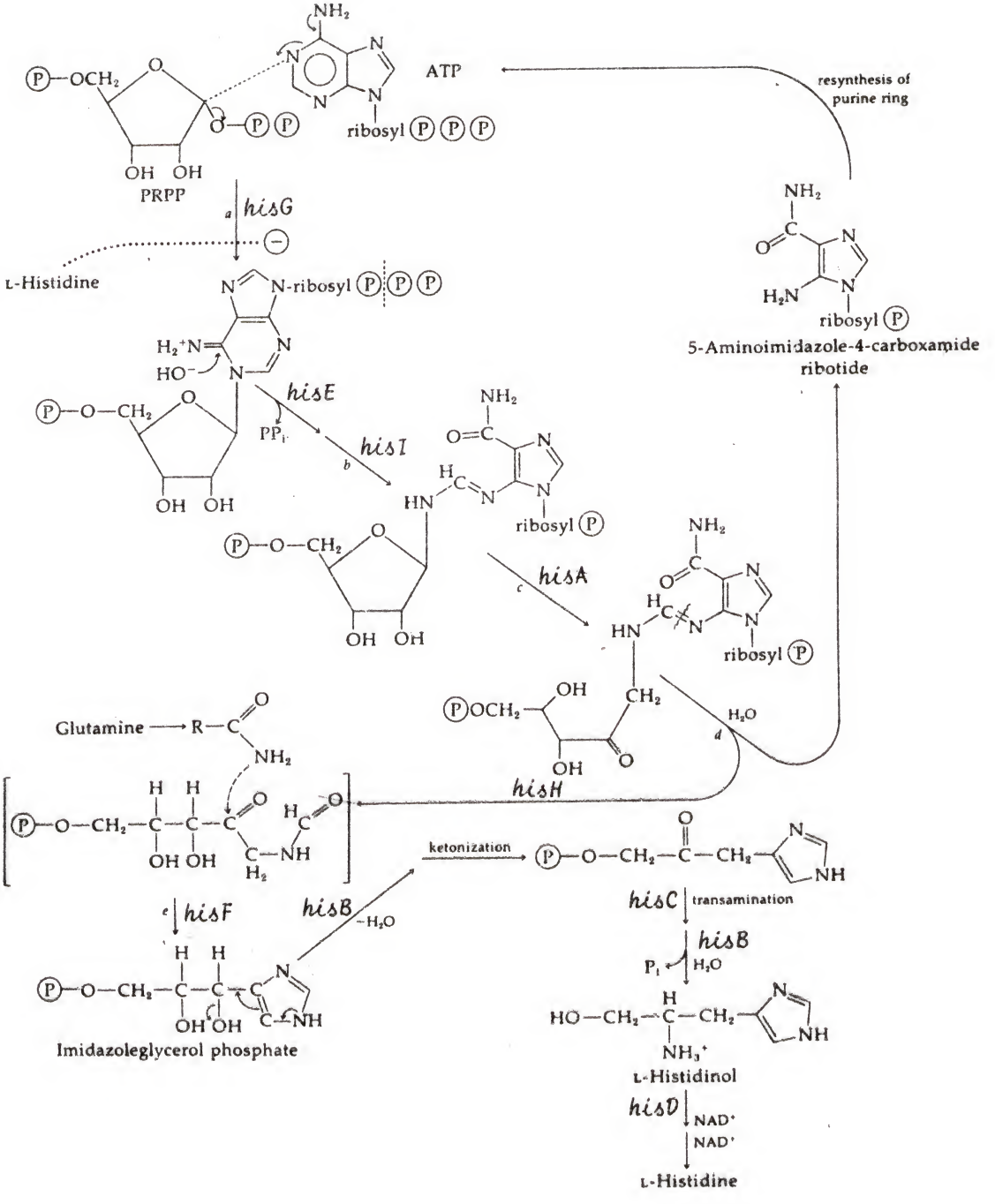


Fig. 1. The pathway of histidine biosynthesis in E. coli and S. typhimurium (cited from Winkler, 1987). hisA to hisI stands for the gene encoding the enzyme for that step. PRPP stands for phosphoribosylpyrophosphate.

tryptophan operon, tyrA (encoding prephenate dehydrogenase), and aroE (encoding enolpyruvyl-shikimate-5-phosphate synthase). The arrangement is 5'-aroF-aroB-aroH-trpE-trpD-trpC-trpF-trpB-trpA-hisH-tyrA-aroE-3' (Henner et al., 1986; Babitzke et al., 1992). In 1971, a "supraoperon" was proposed by Roth and Nester (1971), based on the coordinate expression of hisH and tyrA genes following derepression of the tryptophan operon. Later Nester and Montoya (1976) found that IAP aminotransferase can function in tyrosine and phenylalanine biosynthesis in B. subtilis in addition to the aromatic aminotransferase specified by aroJ. The hisH mutants are auxotrophic for L-histidine, while aroJ mutants remain prototrophic. However, hisH aroJ double mutants are auxotrophic for histidine, phenylalanine and tyrosine. As expected from the in vivo results, purified IAP aminotransferase from B. subtilis was shown to transaminate phenylpyruvate and p-hydroxyphenylpyruvate in vitro (Weigent and Nester, 1976b).

In view of the foregoing physical and functional relationship between hisH and tyrA in B. subtilis, it is intriguing that tyrC in Zymomonas mobilis was found to be adjacent to hisH (Zhao et al., 1993). Are these genes organized as an operon, and does HisH function overlap both histidine and aromatic amino acid biosynthesis? Might these genes comprise part of a still larger complex operon, as in B. subtilis? Z. mobilis is a Gram-negative organism belonging to

the beta subdivision of the proteobacteria (Fox and Jensen, in preparation). At the outset, two evolutionary differences are already known to distinguish Z. mobilis from B. subtilis, with respect to aromatic amino acid biosynthesis. (One) B. subtilis possesses a feedback inhibited prephenate dehydrogenase (specified by tyrA), while Z. mobilis possesses a broad-specificity cyclohexadienyl dehydrogenase (specified by tyrC) which is insensitive to feedback inhibition (Zhao et al., 1993). (Two) In contrast to B. subtilis, Z. mobilis does not possess a tryptophan operon (Eddy et al., 1988). Therefore, if hisH tyrC is part of a complex operon in Z. mobilis, it cannot include more than one or several of the trp genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was used as enriched medium (Silhavy et al., 1984). The minimal medium used for E. coli strains was the M-9 medium (Maniatis et al., 1989). Where indicated, ampicillin was supplemented to the media at 100 μg/ml, tetracycline at 10 μg/ml, thiamine at 17 μg/ml, and amino acids at 50 μg/ml. Medium was solidified with 2% agar.

DNA Manipulation

The subcloning techniques including plasmid purification, restriction enzyme digestion, ligation, and transformation were conducted by standard methods (Maniatis et al., 1989). Restriction enzymes, ligase, calf intestine alkaline phosphatase were purchased from Gibco-BRL or Promega, and were used according to Manufacturer's instructions. Electroporation was conducted by using the Porator from Invitrogen Corp., and following the instruction manual. Southern blot hybridization, using a biotinylated probe, was carried out under stringent conditions according to the instructions of Promega.

Table 1. Bacterial strains and plasmids used in this study.

Strains/plasmids	Genotype/description	Source/ reference
E. coli K-12		
JM83	*ara*Δ(*proAB-lac*) *rpsL* *ϕ80* *lacZ*ΔM15	Gibco-BRL
AT2471	*thi-1* *tyrA4* *relA1* λ^- *spoT1*	CGSC[a]# 4510
UTH780	*hisC780* *malA1*(λ^R), *xyl-5* *rpsL145*, λ^-	CGSC# 5954
DH5α	*supE44*Δ *lacU169* (*ϕ80* *lacZ*ΔM15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*	Gibco-BRL
Z. mobilis (CP4)	Prototroph	L. O. Ingram[b]
Plasmids		
pFBA9	*trpFBA* gene in a 27.4-kb *EcoRI* fragment subcloned into a cosmid	C. Eddy
pC2	*trpC* gene in a 29.4-kb *EcoRI* fragment subcloned into a cosmid	C. Eddy
pC5	*trpC* gene in a 29.1-kb *EcoRI* fragment subcloned into a cosmid	C. Eddy
pF5.8	pUC18 with *trpBA* gene in a 5.8-kb *EcoRI* fragment	C. Eddy
pF20	Cosmid with *trpF* gene in a 34.6-kb *EcoRI* fragment	C. Eddy

Table 1 (continued)

Strains/plasmids	Genotype/description	Source/ reference
pUC18	*lac* Ap^r	Gibco-BRL
pUC19	*lac* Ap^r	Gibco-BRL
pGEM-5zf(+)	*lac* Ap^r	Promega
pJZ5	original clone of *tyrC* isolated from *Z. mobilis* CP4 library	G. S. Zhao
pJZ5b	A derivative of pJZ5 generated by removal of a 3.2-kb *HindIII* fragment	G. S. Zhao
pJG1	1.8-kb *EcoRI*-*NsiI* fragment of pJZ5 subcloned into pUC18 at *EcoRI*-*PstI* site	This study
pJG2	1.1-kb *NcoI*-*StuI* fragment of pJG1 subcloned into pGEM-5zf(+) at *NcoI*-*EcoRV* site	This study
pJG3	2.5-kb *NcoI*-*NcoI* fragment of pJZ5 subcloned into pGEM-5zf(+) at *NcoI* site	This Study
pJG4-a	6-kb *EcoRI*-*HindIII* fragment of pJZ5b subcloned into pUC18 at *EcoRI*-*HindIII* site	This study
pJG4-b	6-kb *EcoRI*-*HindIII* fragment of pJZ5b subcloned into pUC19 at *EcoRI*-*HindIII* site	This study

[a]*Escherichia coli* Genetic Stock Center, Yale University.
[b]Department of Microbiology and Cell Science, University of Florida.

DNA Sequencing and Data Analysis

Plasmid pJG2 was purified by the method described in User Bulletin 18 offered by Applied Biosystems, Inc. Primers were made by the DNA Synthesis Lab of the Institute and Center of Biotechnology Research (ICBR) at the University of Florida. Sequencing was performed by the DNA Core Facility of the ICBR at the University of Florida. The nucleotide sequence and the deduced amino acid sequence were analyzed by using the updated version of sequence analysis software package (GCG) offered by Genetics Computer Group, Inc. (Devereux et al., 1984).

Crude Extract Preparation and Enzyme Assay

Bacterial cultures were grown at 37°C with vigorous shaking in minimal medium supplemented with ampicillin. The cells were harvested in the late-exponential phase of growth by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.0). The cells were broken by sonication using a Ultratip Labsonic System (Lab-Line Instruments, Inc. Melrose Park, IL). The suspension was centrifuged at 150,000xg for 65 min at 4°C. The supernatant fraction was collected and passed through a DG-10 Sephadex column (1.5 x 5.5 cm). The resulting desalted preparation is designated as crude extract.

IAP aminotransferase was assayed by mixing enzyme, α-ketoglutarate (final concentration 10 mM) and histidinol phosphate (final concentration 8.3 mM) in potassium phosphate buffer (50 mM, pH 7.0), incubating at 37°C for the designated amount of time, and then measuring the absorbance at 280 nm of

enolized imidazole acetol phosphate formed enzymatically (Martin et al., 1971). p-Hydroxyphenylpyruvate aminotransferase and phenylpyruvate aminotransferase activities were assayed in 50 mM potassium phosphate buffer (pH 7.0), by using 10 mM α-ketoglutarate and 10 mM L-tyrosine or 10 mM L-phenylalanine as substrates, and following absorbance at 331 nm for p-hydroxyphenylpyruvate or at 320 nm for phenylpyruvate (Whitaker et al., 1982). Other aminotransferase activities were assayed by following the formation of product amino acids by use of high performance liquid chromatography (HPLC) (Bonner and Jensen, 1987). Prephenate dehydrogenase activity was assayed by following the appearance of NADH on a spectrophotofluometer (excitation at 340 nm and emission at 460 nm) at 37°C with saturating concentrations of NAD^+ (1 mM) and prephenate (2 mM) (Zhao, 1991). Extinction coefficients of 5,310 for imidazole acetol phosphate, 17,500 for phenylpyruvate, and 32,850 for p-hydroxyphenylpyruvate were used to calculate the concentration of each product (Martin et al., 1971; Whitaker et al., 1982). Specific activity of the enzyme was defined in terms of micromole of product per min per mg of protein at 37°C. Protein concentration was determined by the method of Bradford (1976).

Purification of the Cloned Imidazole Acetol Phosphate Aminotransferase from E. coli UTH780

E. coli UTH780 carrying the plasmid pJG3 was grown in 6 liters of minimal medium supplemented with ampicillin and thiamine at 37°C in a gyrotory shaker up to the late

exponential phase of the growth. The cell pellet was harvested by centrifugation and washed once with buffer A (potassium phosphate buffer, 50 mM, pH 7.0; DTT, 1.0 mM; β-mercaptoethanol, 1.4 mM; PLP, 0.05 mM), then resuspended in the same buffer and disrupted by sonication. After centrifugation (150,000xg for 65 min at 4°C), the supernatant was dialyzed overnight against buffer A, and then applied to a DEAE-cellulose column (2.5 x 55 cm) equilibrated with buffer A. The column was washed with 700 ml of buffer A, and then eluted with 1,800 ml of buffer A containing a linear gradient of KCl from 0 mM to 200 mM. Fractions of 8 ml were collected, and those showing high IAP aminotransferase activity were pooled and concentrated by means of an Amicon PM-10 membrane. The concentrated preparation was dialyzed against buffer A overnight, and applied to a hydroxylapatite column (1.5 x 51 cm) equilibrated with buffer A. After washing with 400 ml of buffer A, the column was eluted with 1,000 ml of buffer A containing a linear gradient of potassium phosphate from 10 mM to 400 mM. Fractions of 5 ml were collected and those showing high IAP aminotransferase activity were pooled and concentrated as described before.

A native polyacrylamide gel electrophoresis, using the foregoing preparation, was performed as described by Orr et al. (1972). After electrophoresis, a part of the gel was stained with Coomassie blue. The portion of the unstained gel which corresponded to the bands visualized on the stained gel

were cut off separately and minced in buffer A, then incubated at 37°C for an hour. The gel was removed by centrifugation at 10,000xg by means of microfilterfuge tube. The preparation recovered could be used for further enzyme analysis.

Amino Acid Sequencing of the Cloned Imidazole Acetol Phosphate Aminotransferase

The purified enzyme preparation was denatured by SDS and subjected to polyacrylamide gel electrophoresis (Laemmli, 1970). The protein was transferred to a polyvinylidene difluoride membrane by a protein mini blotting apparatus and sequenced using an Applied Biosystems 407A Protein Sequencer with On-line 120A PTH-Analyzer at the Protein Core Facility of the ICBR at the University of Florida.

Molecular Mass Determination

The molecular mass of the native enzyme was estimated by gel filtration using a Sephadex G-200 column (2.5 x 98 cm) which had been previously equilibrated with buffer A and eluted with the same buffer. The column was calibrated by chymotrypsinogen, 25,000; albumin, bovine serum, 66,000; alcohol dehydrogenase, 150,000; and β-amylase 200,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the subunit molecular mass of the enzyme. α-Lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; albumin, bovine serum, 67,000; phosphorylase 94,000 were used as molecular mass standards.

Biochemicals and Chemicals

Histidinol phosphate, α-ketoglutarate, pyridoxal 5'-phosphate (PLP), β-mercaptoethanol, ampicillin, tetracycline, thiamine, Sephadex G-200, and amino acids were purchased from Sigma Chemical Company; dithiothreitol (DTT) was from Research Organics, Inc.; DEAE-cellulose was from Whatman; hydroxylapatite was from BioRad. Molecular mass standards for gel filtration and for SDS-PAGE were from Sigma Chemical Company and Pharmacia Fine Chemicals, respectively. Growth medium components and agar were from Difco.

RESULTS AND DISCUSSION

Identification of the Gene Upstream of tyrC

Zhao et al. (1993) identified a partial open reading frame of 539 bp located directly upstream of tyrC (encoding cyclohexadienyl dehydrogenase) in Z. mobilis. Amino acid sequence comparison with other sequences in the database (GenBank and Embl) indicated that this partial sequence showed the highest identity with IAP aminotransferase from B. subtilis (34.7% identity).

It was anticipated that the original clone obtained by Zhao et al. (1993) would contain an intact hisH gene. This was tested by using this clone, pJZ5, for functional complementation. E. coli UTH780, a hisC mutant (lacking IAP aminotransferase activity), was used as a recipient, pJZ5 and pUC18 control were transformed into E. coli UTH780, and spread on M-9 medium plus ampicillin. After 16 hours, ampicillin-resistant transformants were found on E. coli UTH780/pJZ5 containing plates, while there were no colonies on UTH780/pUC18 control plates. Plasmids purified from the ampicillin-resistant transformants had the same size and pattern as pJZ5 on agarose gel. A second transformation of E. coli UTH780 with these purified plasmids also conferred histidine prototrophy.

Subcloning experiments localized hisH to the NcoI-NcoI fragment. When the EcoRI-NsiI fragment was subcloned into pUC18, the resulting plasmid, designated as pJG1, was unable to complement E. coli UTH780 to histidine prototrophy (Fig. 2). These data confirmed that hisH was just upstream of tyrC, and indicated that cleavage at the NsiI site disrupted the integrity of the structural gene. This was confirmed later by the sequencing data. Southern blot hybridization showed that a labeled 0.6-kb StuI-StuI fragment of pJG3 hybridized with a 0.6-kb fragment of Z. mobilis chromosomal DNA digested with StuI, but did not hybridize with E. coli chromosomal DNA digested with the same enzyme (Fig. 3).

Z. mobilis IAP aminotransferase expression in E. coli was measured directly (Table 2). Histidine auxotroph UTH780 exhibited the expected absence of IAP aminotransferase activity. The his^+ E. coli tyrosine auxotroph AT2471 possessed a specific activity of 42.1 nmol/minxmg for IAP aminotransferase. The enzyme activity increased 3-fold when the larger plasmid pJZ5 insert was trimmed to the smaller insert size of pJG3. Both subclones, UTH780/pJG4-a and UTH780/pJG4-b (the same insert fragment subcloned into pUC18 and pUC19, respectively), showed enzyme activity. However, UTH780/pJG4-a had 2-fold higher activity than UTH780/pJG4-b. Thus the promoter region of hisH could be recognized by E. coli RNA polymerase.

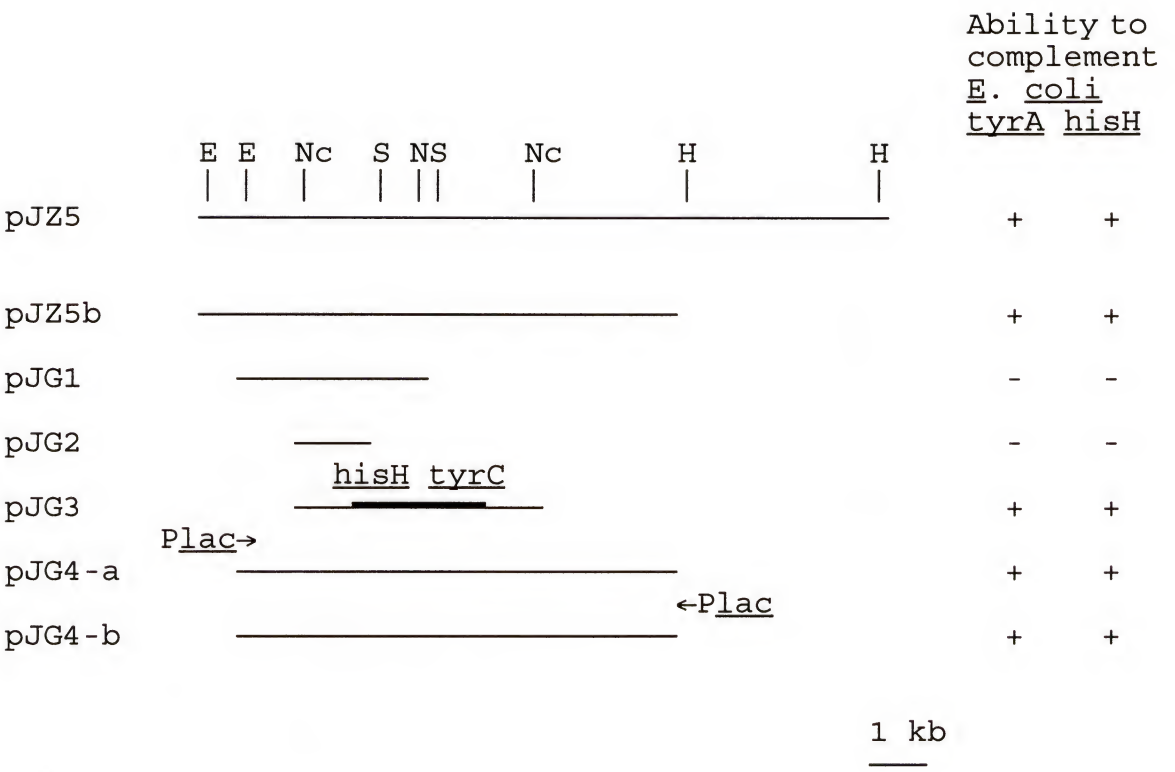


Fig. 2. Physical location of the Z. mobilis genes encoding imidazole acetol phosphate aminotransferase (hisH) and cyclohexadienyl dehydrogenase (tyrC). Abbreviations: E, EcoRI; Nc, NcoI; S, StuI; N, NsiI; H, HindIII; Plac, lac promoter. Heavy bar stands for both hisH and tyrC.

Fig. 3. Southern blot hybridization. A labeled 0.6-kb StuI fragment of pJG3 was hybridized with a 0.6-kb fragment of a StuI digest of Z. mobilis chromosomal DNA. Lane 1: Z. mobilis chromosomal DNA digested with StuI. Lane 2: E. coli chromosomal DNA digested with StuI.

Table 2. Expression of the Z. mobilis imidazole acetol phosphate aminotransferase and cyclohexadienyl dehydrogenase genes in E. coli.

Strain/plasmid	Enzyme activity (milli units)	
	IAT	CDH
UTH780/pUC18	-0-	ND
AT2471/pUC18	42.1	-0-
UTH780/pGEM-5zf(+)	-0-	ND
UTH780/pJZ5	76.5	ND
AT2471/pJZ5	ND	14.4
UTH780/pJG3	227.4	ND
AT2471/pJG3	ND	71.8
UTH780/pJG4-a	104.7	ND
AT2471/pJG4-a	ND	103.0
UTH780/pJG4-b	50	ND
AT2471/pJG4-b	ND	23.1

Enzyme activity was determined using crude extract. Prephenate was used for CDH assay.
Abbreviations: IAT, imidazole acetol phosphate aminotransferase; CDH, cyclohexadienyl dehydrogenase; ND, not determined.

Nucleotide Sequence of the Imidazole Acetol Phosphate Aminotransferase Gene

The complete nucleotide sequence of the imidazole acetol phosphate aminotransferase gene along with its upstream regions is shown in Fig. 4. The structural gene, 1110 bp in length, begins at codon ATG and terminates at codon TAA. The N-terminal amino acid sequence, by using the sample cut from the major band on SDS-PAGE, was determined to be Met-Thr-Ala-Ala-Pro-Glu (MTAAPE), a sequence which was identical to that

Fig. 4. Nucleotide sequence of the imidazole acetol phosphate aminotransferase gene along with its upstream region. The deduced amino acid sequence of the gene is given beneath the corresponding codons. The presumed -35 region, -10 region, and ribosome binding site (RBS) region are underlined and labeled. The residues which correspond to the PLP binding site (PBS) and restriction site are also underlined and labeled.

```
        10                  30                  50
ATAAAAAATATAAAAAACAGTTTATCGCTAAAAAAATCGGCAATTTTACTCTATTAGGAA
        70                  90                 110
ATACCAAAAAATAAAAATTAAAGCCTTTTTAAACAAAATCATTAAAGAAAATTTAGTTTT
       130                 150                 170
TTAATTTTTTATTATAAAAAACAAGAAAAACACAACCCAATAAACCAAATCAATCTATTT
       190                 210                 230
TCTGCTTGGACCAAAAAGCTGTAAAATTTTTTATAAATTATGATACAAAATCAGTCACTT
 -35   250                -10 270                 290
TGGTTCTAAAATAAAGAAAAGACTATTAAAATATCGAGGATAGCCTTTAACGCGCGCCATT
       310          RBS    330                 350
ATTATCCGGATTTTTATCAAAGAGCTATTATGACTGCTGCACCTGAACTTCGCCCGAAAT
                              M   T   A   A   P   E   L   R   P   K   S
       370                 390                 410
CTTGGATCGACAGTATTGCGCCTTACATTCCGGGGTCATCAAAGACGCTGGATGGCCGTC
  W   I   D   S   I   A   P   Y   I   P   G   S   S   K   T   L   D   G   R   P
       430                 450                 470
CGGCTGTCAAACTTTCATCTAATGAAAACCCTTTGGGAACCAGCCTAAAGGCGAAAGAAG
  A   V   K   L   S   S   N   E   N   P   L   G   T   S   L   K   A   K   E   A
       490                 510                 530
CCTATCGGGAAGCCATAGACTCCCTTTCCCTGTATCCTGACAGTGGGGCAACCGCTCTGC
  Y   R   E   A   I   D   S   L   S   L   Y   P   D   S   G   A   T   A   L   R
       550                 570                 590
GTGAGGCCATCGGCGCTTGCTATAATCTTGATCCGGCTCGGATTATTCACGGCACCGGAT
  E   A   I   G   A   C   Y   N   L   D   P   A   R   I   I   H   G   T   G   S
       610                 630                 650
CGGATGAAATCCTGCATTTGGCCGCCGGTGCCTATGCCGGACAAGATGACGAGGTTTTAT
  D   E   I   L   H   L   A   A   G   A   Y   A   G   Q   D   D   E   V   L   Y
       670                 690                 710
ATCCTCGCTATAGTTTTTCAGTTTACCCGCTGGCTGCCAGACGCGTAGGCGCAACACCGG
  P   R   Y   S   F   S   V   Y   P   L   A   A   R   R   V   G   A   T   P   V
       730                 750                 770
TAGAAGCACCAGATGATGACTATCGTTGTTCTGTTGATGCCTTGTTAAAGGCTGTCACGC
  E   A   P   D   D   D   Y   R   C   S   V   D   A   L   L   K   A   V   T   P
       790                 810                 830
CACGGACACGGGTTGTCTTTATTGCCAATCCGAATAACCCGACGGGAACATGGATTACCC
  R   T   R   V   V   F   I   A   N   P   N   N   P   T   G   T   W   I   T   R
       850                 870                 890
GTGCAGAAGTTGAAAAACTGCATAACGGCCTTCCCCGTAACTGCTTATTGGTTATCGATC
  A   E   V   E   K   L   H   N   G   L   P   R   N   C   L   L   V   I   D   Q
 StuI  910                 930                 950
AGGCCTATGCTGAATATCTTGATCCTGAATGTGATGATGGCGCGTTAGCACTCGCCAAAA
  A   Y   A   E   Y   L   D   P   E   C   D   D   G   A   L   A   L   A   K   N
       970                 990 PBS            1010
ACACAAAAAATGTGCTGGTAACGCGGACATTTTCTAAAATCTACGGATTGGCCGCTGAAA
  T   K   N   V   L   V   T   R   T   F   S   K   I   Y   G   L   A   A   E   R
      1030                1050                1070
GAATTGGCTGGGCTTATGCTTGCCCTGAAATCATTGATGCCCTTAACCGGATACGGGCAC
  I   G   W   A   Y   A   C   P   E   I   I   D   A   L   N   R   I   R   A   P
      1090                1110                1130
CCTTCAATGTCACGATTGCGGGACAAAAGGCAGCGGTCGCCGCTTTGGAAGATCAGGCGT
  F   N   V   T   I   A   G   Q   K   A   A   V   A   A   L   E   D   Q   A   F
      1150                1170                1190
TCATCCAGAACAGCTTCAAACATAGCAAAAAAGTGGCGTGGCTGGTTGAAAACCAGATGG
  I   Q   N   S   F   K   H   S   K   K   V   A   W   L   V   E   N   Q   M   A
      1210                1230                1250
CGCTTTTAAGTAATGTTGGCATTCGTGTTATCCCGTCCTCTGCCAATTTTACTCTACTGC
  L   L   S   N   V   G   I   R   V   I   P   S   S   A   N   F   T   L   L   L
      1270                1290                1310
TGTTTGAAGGCAGCCTGACGGCTAAAACCGCCTATAAAGCCTTGATGGATCACGGCTATA
  F   E   G   S   L   T   A   K   T   A   Y   K   A   L   M   D   H   G   Y   T
      1330                1350   NsiI         1370
CCACCCGTTGGTTGCCGGGACAGCGCCTTCCTCATGCATTACGTATCACTATCGGCAGTG
  T   R   W   L   P   G   Q   R   L   P   H   A   L   R   I   T   I   G   S   E
      1390                1410                1430
AAAAACATATGCAGGATGTCGCTGGTATTTTAACTTCCTTGGTTAGGCAGGCGCTCTAA
  K   H   M   Q   D   V   A   G   I   L   T   S   L   V   R   Q   A   L   *
```

deduced from the nucleotide sequence (Fig. 4). The deduced amino acid sequence yields a protein of 370 residues with a molecular mass of 40,631 Da. This agrees well with the value of 40,000 Da determined for the purified enzyme by SDS-PAGE (Fig. 5C).

The G+C content of the gene is 50.1%, which falls into the range 47%-50% for the Z. mobilis genome (Montenecourt, 1985). By comparing with the other known sequences from Z. mobilis (Pond et al., 1989), the sequence AAAGAG, located 5 bp upstream of the start codon may serve as the ribosome binding site (Shine and Dalgarno, 1974). E. coli-like sigma-70 promoter sequence (TTGGTT-18-TATTAA) is located 60 bp upstream of the start codon, the deduced 5'end transcribed region is A+T rich. The codon usage of hisH exhibits a different bias from Z. mobilis genes which encode high-abundance proteins (Table 3) (Pond et al., 1989). For instance, nine codons (GGG GTA AGG AGA CGG ATA ACA CTA CCC) in Table 3 are never (5) or rarely (4) used in high-abundance proteins, but used to a significantly greater extent in the low-abundance proteins. This is consistent with expectations that the low-abundance tRNA species in the cell may limit gene expression.

Purification of the Cloned Imidazole Acetol Phosphate Aminotransferase, and its Inseparability from the Aromatic Aminotransferase

The Z. mobilis enzyme was purified from the clone UTH780/pJG3 in E. coli by fractionation employing two chromatography steps (DEAE-cellulose, hydroxylapatite) (Table 4). One major protein band and one minor protein band were

Fig. 5. Polyacrylamide gel electrophoresis of the cloned IAP aminotransferase from E. coli UTH780. A) SDS-PAGE of the protein sample after different purification steps. The protein samples were run in a 12% gel and stained with Coomassie blue. Lane 1: the collected fractions after gel filtration; Lane 2: the collected fractions after hydroxylapatite chromatography; Lane 3: the collected fractions after DEAE-cellulose chromatography; Lane 4: crude extract; Lane 5: molecular weight standards; B) Native gel electrophoresis of the purified IAP aminotransferase from E. coli UTH780. The protein samples were run in a 12% gel and stained with Coomassie blue; C) SDS-PAGE of the protein sample from the eluted fraction of the top band from the native gel presented in (B). Lane 1: molecular weight standards; Lane 2,3: the eluted fraction.

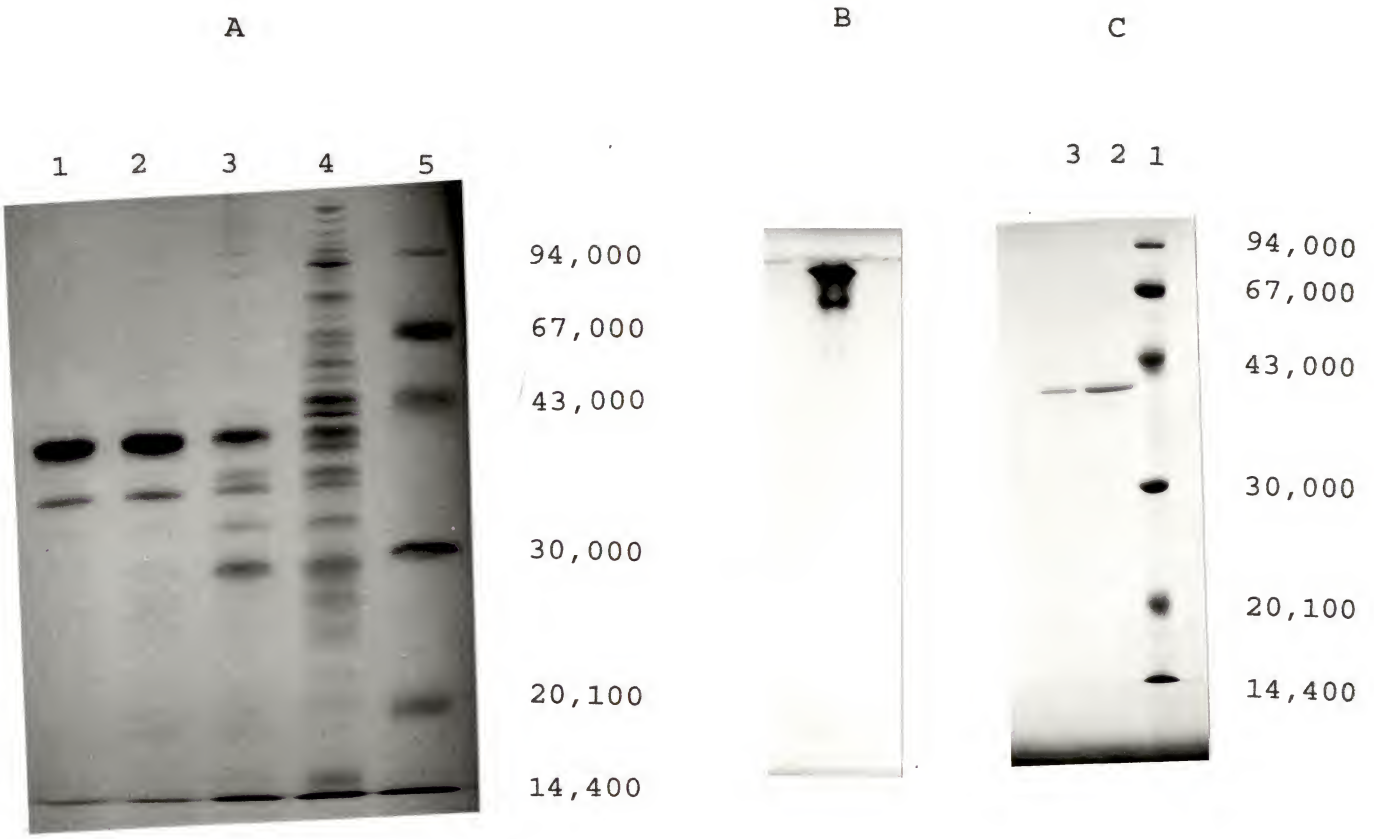

A
B
C
1 2 3 4 5
94,000
67,000
43,000
30,000
20,100
14,400
3 2 1
94,000
67,000
43,000
30,000
20,100
14,400

Table 3. Comparison of codon usage of *hisH* with other genes in *Z. mobilis*.

Amino acid	Codon	Frequency (mol% codon usage)				
		hisH	*tyrC*	*phoC*[a]	*Z. mobilis* combined[a]	*E. coli* combined[a]
Gly	GGG	0.5	1.0	0.4	0	0.6
	GGA	1.9	1.0	1.1	0.1	0.4
	GGT	0.5	2.0	1.5	5.8	3.8
	GGC	3.0	0.7	3.4	2.3	3.1
Glu	GAG	0.5	1.4	0	0.4	1.8
	GAA	4.3	3.1	4.2	5.1	4.9
Asp	GAT	4.1	5.4	6.1	3.4	2.5
	GAC	1.4	2.7	1.1	2.6	3.0
Val	GTG	0.5	1.4	0.8	0.5	2.2
	GTA	0.8	1.4	0	<0.1	1.8
	GTT	2.7	2.0	1.5	5.6	2.9
	GTC	1.6	2.7	0.8	2.6	1.2
Ala	GCG	2.4	4.1	3.0	0.8	3.2
	GCA	2.4	3.1	3.4	2.4	2.3
	GCT	3.8	3.1	2.3	8.2	2.6
	GCC	4.9	2.4	2.7	3.0	2.2
Arg	AGG	0.3	0.3	0	0	<0.1
	AGA	0.5	0.7	0.8	0	<0.1
	CGG	1.9	1.7	1.1	<0.1	0.2
	CGA	0	0	0.8	0	0.2
	CGT	2.2	1.7	0.4	2.0	3.1
	CGC	1.1	0	3.0	1.3	2.0
Ser	AGT	1.4	0.3	0.8	0.2	0.3
	AGC	1.1	2.0	3.4	1.3	1.4
	TCG	0.3	2.4	0.8	0.4	0.6
	TCA	1.1	0	1.5	0.3	0.4
	TCT	1.4	0.3	0.4	1.1	1.3
	TCC	1.1	0.3	1.5	1.4	1.5
Lys	AAG	1.1	0.7	0.8	2.7	1.3
	AAA	3.5	4.1	3.8	3.9	4.1
Asn	AAT	2.2	2.4	1.9	1.6	1.0
	AAC	2.2	0.3	0.8	3.1	2.8
Met	ATG	1.1	2.0	1.9	2.7	2.8
Ile	ATA	0.5	0.7	0.4	0	0.2
	ATT	3.0	4.1	3.4	1.3	2.2
	ATC	2.7	3.1	2.3	3.4	3.7
Thr	ACG	1.6	1.0	1.5	1.3	0.8
	ACA	1.4	2.0	1.1	<0.1	0.3
	ACT	1.1	0.3	1.5	0.6	1.1
	ACC	1.9	1.4	1.9	3.8	2.4
Trp	TGG	1.4	0.7	1.5	0.6	0.7

Table 3. (continued)

Amino acid	Codon	Frequency (mol% codon usage)				
		hisH	tyrC	phoC[a]	Z. mobilis combined[a]	E. coli combined[a]
Cys	TGT	0.5	0.3	0.4	0.2	0.4
	TGC	0.8	1.0	1.5	0.9	0.5
Tyr	TAT	3.2	2.4	1.9	1.6	1.0
	TAC	0.8	0.3	0.4	0.7	1.5
Leu	TTG	2.4	1.4	1.5	1.5	0.9
	TTA	1.9	2.7	2.3	0.2	0.7
	CTG	3.0	2.4	1.1	3.9	6.8
	CTA	0.5	0.3	0.4	0	0.2
	CTT	2.4	1.7	3.4	1.9	0.8
	CTC	0.5	1.7	1.5	1.7	0.8
Phe	TTT	1.4	1.7	1.1	0.5	1.3
	TTC	0.8	0.7	1.5	2.6	2.2
Gln	CAG	1.9	0.7	1.1	1.6	3.2
	CAA	0.5	2.0	3.4	0.1	1.0
His	CAT	1.4	3.1	3.4	1.1	0.7
	CAC	0.5	1.7	0.8	1.3	1.2
Pro	CCG	2.7	0.7	0.8	2.9	2.5
	CCA	0.5	0.7	1.1	0.4	0.7
	CCT	2.2	2.7	0.8	0.8	0.5
	CCC	0.5	1.4	2.3	0.2	0.3
End	TGA	0	0	-	-	-
	TAA	0.3	0.3	-	-	-
	TAG	0	0	-	-	-

[a] Data from Pond et al., 1989. "Z. mobilis combined" stands for average of Z. mobilis gap, pgk, adhB, and pdc. "E. coli combined" stands for average of 52 proteins from E. coli.

Table 4. Purification of the cloned Z. mobilis imidazole acetol phosphate aminotransferase from E. coli UTH780/pJG3.

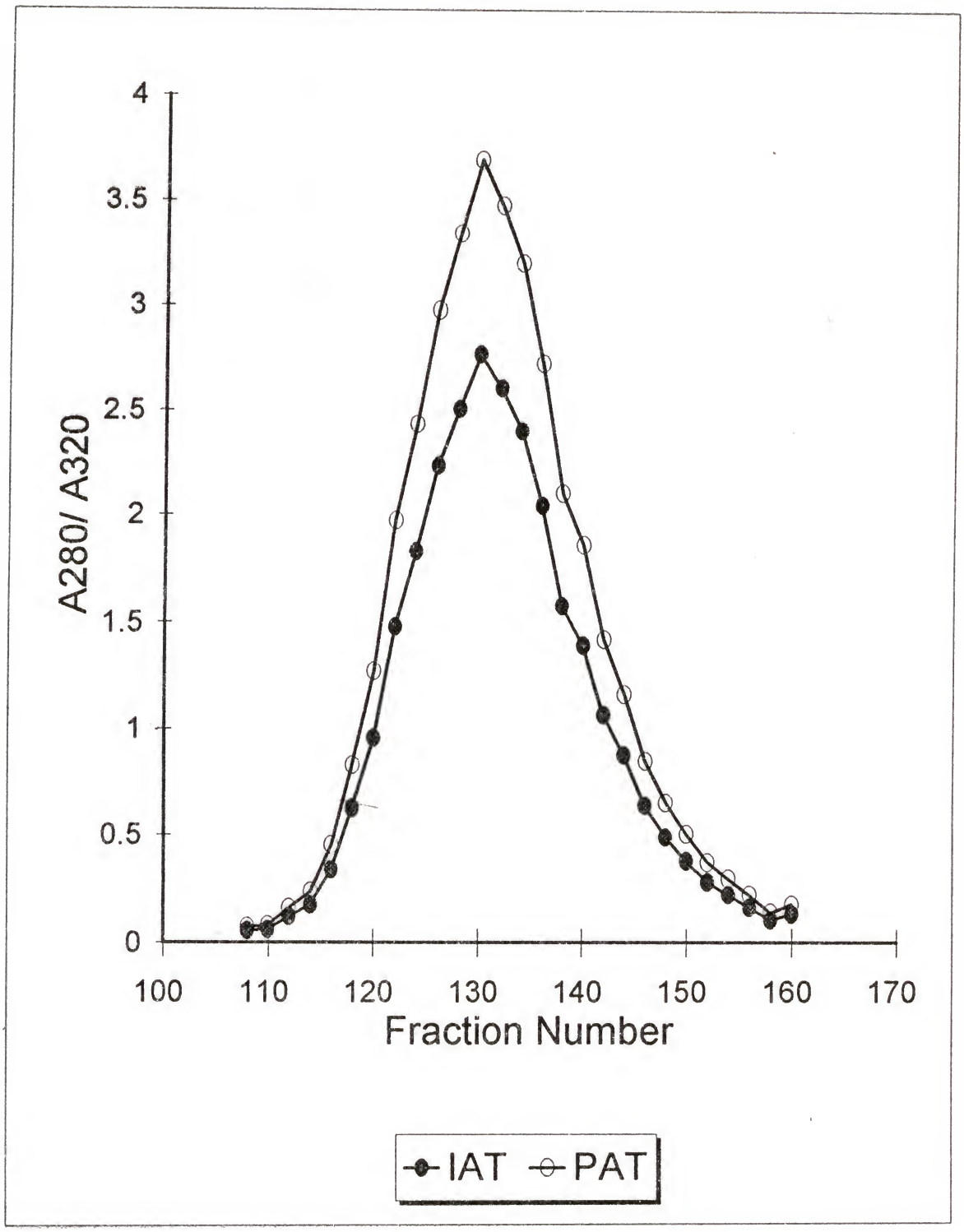
Step	Total protein (mg)	SA		IAT/PAT Ratio	Purification factor
		IAT	PAT		
Crude Extract	818	0.52	2.15	0.24	1
DEAE-cellulose	95.2	3.54	8.67	0.41	6.8
Hydroxyl-apatite	20.9	18.24	29.93	0.64	35

Abbreviations: SA, specific activity; IAT, imidazole acetol phosphate aminotransferase; PAT, phenylpyruvate amino-transferase.

visualized by SDS-PAGE (Fig. 5A). The molecular mass of the major band was estimated to be 40,000 Da, in good agreement with that deduced from DNA sequence. The molecular mass of the native enzyme is 85,000 Da, as determined by gel filtration on Sephadex G-200. The native enzyme is therefore a homodimer.

The fractions which contained IAP aminotransferase activity also exhibited aromatic aminotransferase activity (Fig. 6). The purified enzyme preparation from the hydroxylapatite column was loaded on a native polyacrylamide gel (Fig. 5B). The protein corresponding to the top band (see methods) was eluted and found to exhibit imidazole acetol phosphate, phenylpyruvate and p-hydroxyphenylpyruvate aminotransferase activities. A sample of the same eluate also

Fig. 6. Elution profile of the enzyme assayed as imidazole acetol phosphate aminotransferase (IAT), and phenylpyruvate aminotransferase (PAT) after hydroxylapatite chromatography.


4
3.5
3
2.5
2
1.5
1
0.5
0
A280/ A320
100
110
120
130
140
150
160
170
Fraction Number
IAT
PAT

gave a single 40,000 Da band on SDS-PAGE (Fig. 5C), thus indicating successful removal of the minor protein from the sample.

To confirm further that the minor protein was not responsible for the aromatic aminotransferase activity observed, N-terminal amino acid sequencing of the minor protein was carried out. The resulting "MKVAV" sequence did not correspond with that of tyrB "VFQKV", aspC "MFENI", and ilvE "MTTKK" of E. coli. These three E. coli enzymes would be the only possible extraneous source of aromatic amino-transferase activity (Gelfand and Steinberg, 1977). These data show that IAP aminotransferase possesses broad substrate specificity which includes aromatic keto/amino acids.

Properties of Imidazole Acetol Phosphate Aminotransferase

Heat Stability

The purified enzyme showed relatively good tolerance to elevated temperatures. Both IAP amino-transferase and phenylpyruvate aminotransferase activities remained unchanged when the enzyme was treated at 50°C, but decreased sharply and in parallel when incubated above 55°C for 2 min (Fig. 7).

Freeze-Thaw Stability

The purified enzyme is unstable to freeze-thaw treatment. After freezing at -20°C overnight and thawing on ice, 98% of the IAP aminotransferase and phenylpyruvate aminotransferase activities were lost, while after freezing at -80°C overnight and thawing on ice, 58% of both activities were lost. In

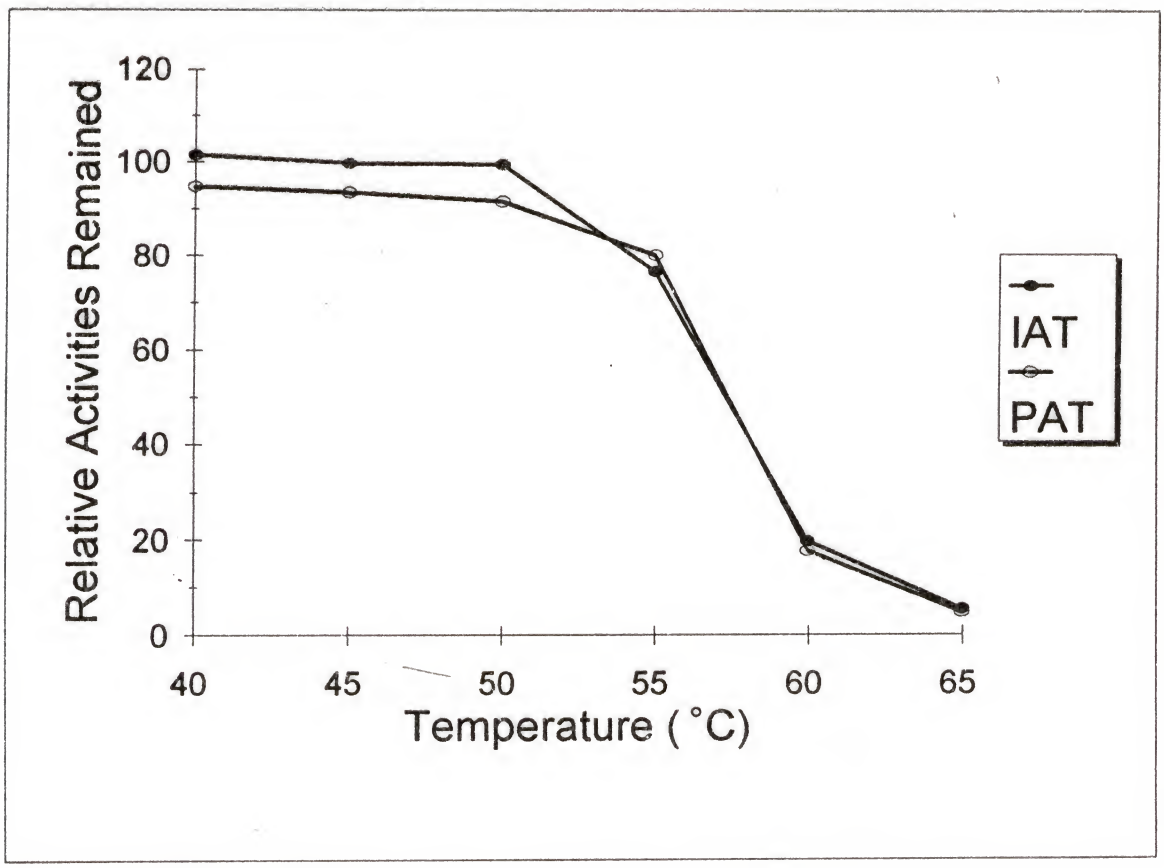


Fig. 7. Heat stability of the purified enzyme assayed as imidazole acetol phosphate aminotransferase (IAT), and phenylpyruvate aminotransferase (PAT). The enzyme preparations were incubated at the indicated temperature for 2 min, then kept on ice. The assay conditions were as described under Materials and Methods section. A relative activity of 100 is the activity assayed in control sample which were unheated. The control sample has 6.2 μmol/minxmg for IAP aminotransferase, and 19.8 μmol/minxmg for phenylpyruvate aminotransferase.

contrast, 100% of both activities remained after several months at -80°C in 10% (v/v) glycerol.

Requirement for PLP

When the purified enzyme preparation was dialyzed against 50 mM potassium phosphate buffer overnight to remove α-ketoglutarate, PLP, and β-mercaptoethanol, both IAP aminotransferase and phenylalanine aminotransferase activities were unaltered. Enzyme activity was neither dependent upon nor stimulated by the presence of PLP in the reaction buffer. However, when the purified enzyme preparation was dialyzed against 20 mM potassium phosphate buffer containing 3 mM hydroxylamine, and then dialyzed against the same buffer without hydroxylamine to remove the pyridoxamine generated, no enzyme activity could be detected in this PLP-resolved enzyme preparation. Incubation of the PLP-resolved enzyme preparation with 0.1 mM PLP for 15 min at 37°C restored about 17% of the original activities with either histidinol phosphate or phenylpyruvate. These results indicated that PLP binds tightly to the enzyme. Removal of the cofactor apparently triggers irreversible denaturation. The presence of 10% glycerol, which stabilizes the enzyme during storage, did not stabilize the apo-enzyme resolved of cofactor.

Effect of pH on Enzyme Activity

The purified enzyme exhibited a similar broad pH range between pH 6.0-8.0 when different substrates were used (Fig. 8). However, utilization of histidinol phosphate was favored

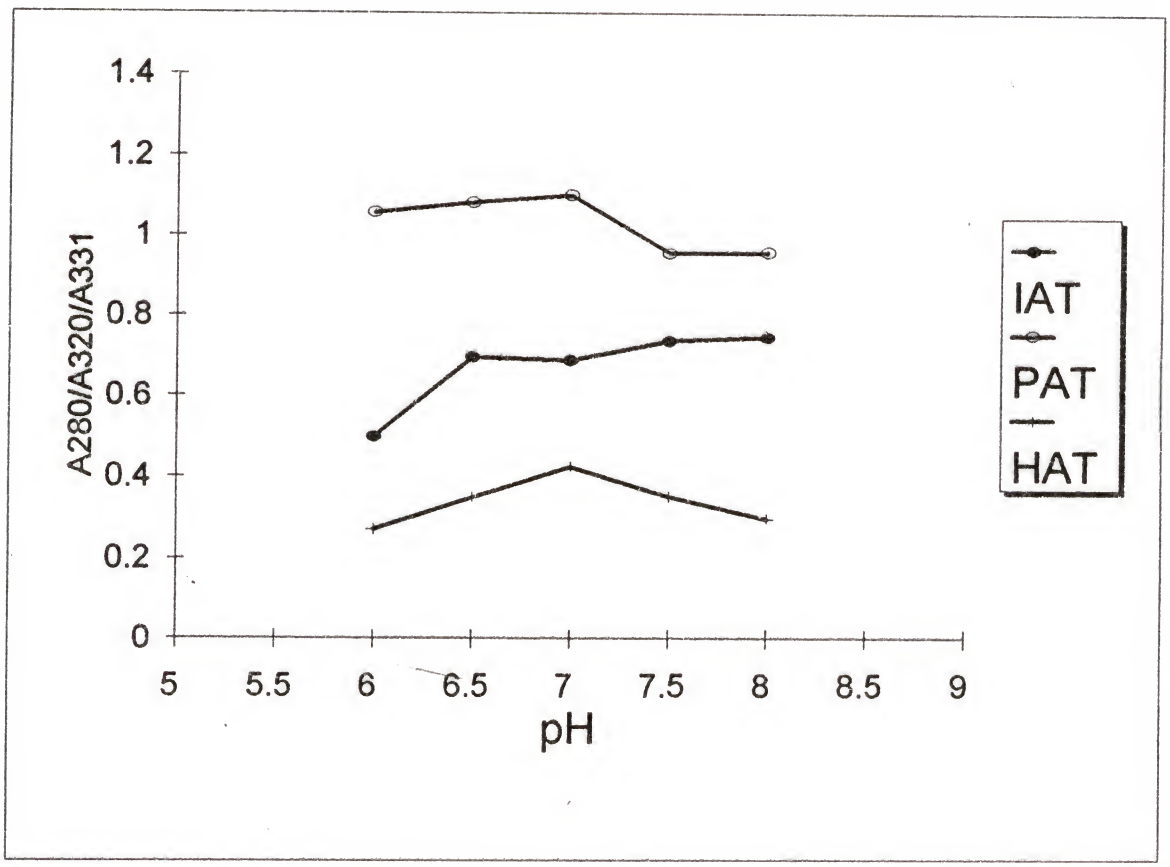


Fig. 8. Effect of pH on purified enzyme activity. Potassium phosphate buffer, pH: 6.0-8.0 was used. See Materials and Methods for enzyme assay. Abbreviations: IAT, imidazole acetol phosphate aminotransferase; PAT, phenylpyruvate aminotransferase; HAT, p-hydroxypheylpyruvate amino-transferase.

by a somewhat higher pH than that observed with phenylalanine or tyrosine as amino-donor substrates.

The Substrate Specificity of the Purified Enzyme

The specific activities of the purified enzyme, when different substrates were used, are listed in the Table 5. In addition to high activities when histidinol phosphate, phenylalanine and tyrosine were used as amino donors, histidine was also found to be an effective substrate. The enzyme has only very low activity as an aspartate aminotransferase or alanine aminotransferase, judging from the oxaloacetate and pyruvate in combination with histidinol phosphate. Phospho-serine, a

Table 5. Specific activity of the purified enzyme using different substrate combinations.

Histidinol phosphate		α-Ketoglutarate	
α-Keto-glutarate	7.0	Phenyl-alanine	11.6
p-Hydroxy-phenylpyruvate	7.11	Tyrosine	11.6
Phenyl-pyruvate	0.56	Histidine	1.13
Oxalo-acetate	0.1	Leucine	0.06
Pyruvate	0.25	Phospho-serine	0

See Materials and Methods section for enzyme assay. All activities were assayed by following the formation of product amino acids using HPLC.

phosphorylated intermediate of a biosynthetic pathway (like histidinol phosphate), was not utilized at all by IAP aminotransferase.

Kinetics of the Purified Enzyme

A series of kinetic analyses were carried out. Double-reciprocal plots of initial velocity as a function of varied concentration of one substrate when the other substrate was maintained at various fixed concentrations gave a family of parallel lines (Fig. 9). These experimental results conform with expectations for a Ping Pong Bi Bi type of reaction mechanism, as has been demonstrated for many other aminotransferases (Sung et al., 1990). The K_m, and V_{max} values for different substrates calculated from the secondary plots of intercept versus reciprocal concentrations of the fixed substrate (Fig. 10) have been listed in Table 6. The markedly higher affinity for histidinol phosphate than for any of the alternative substrates is consistent with its probable major role as an enzyme of histidine biosynthesis.

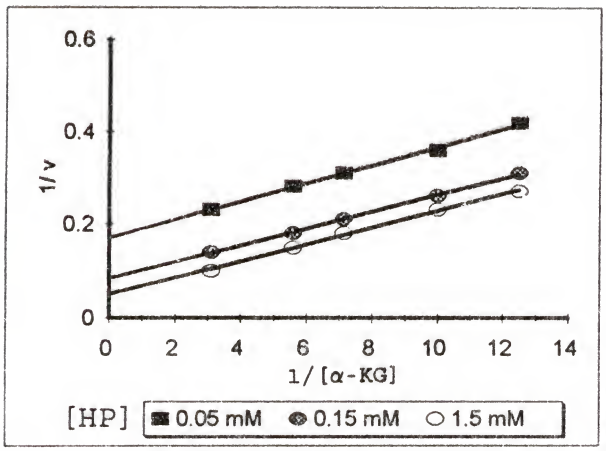
Possible Linkage of hisH-tyrC with Genes of Tryptophan Biosynthesis

The plasmid pF5.8 and cosmids pFBA9, pC2, pC5, and pF20 were transformed into E. coli UTH780 and AT2471 by electroporation, none of the transformants obtained complemented the hisC and tyrA deficiencies. Thus, linkage of hisH-tyrC with trpFBA and trpC has been eliminated. Linkage of trpD and/or trpE have not been eliminated.

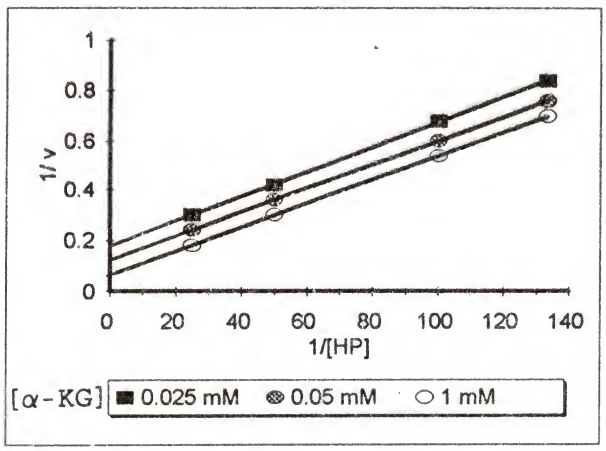
Fig. 9. Double reciprocal plots of initial velocity as a function of one substrate concentration (mM) at several fixed concentrations (mM) of the second substrate. A) Enzyme assayed as IAP aminotransferase. A-1: [α-ketoglutarate] (α-KG) is varied, [histidinol phosphate] (HP) is fixed; A-2: [HP] is varied, [α-KG] is fixed. B) Enzyme assayed as phenylpyruvate aminotransferase. B-1: [α-KG] is varied, [phenylalanine] (Phe) is fixed; B-2: [phe] is varied, [α-KG] is fixed. C) Enzyme assayed as p-hydroxyphenylpyruvate aminotransferase. C-1: [α-KG] is varied, [tyrosine] (Tyr) is fixed; C-2: [Tyr] is varied, [α-KG] is fixed.

A

A-1

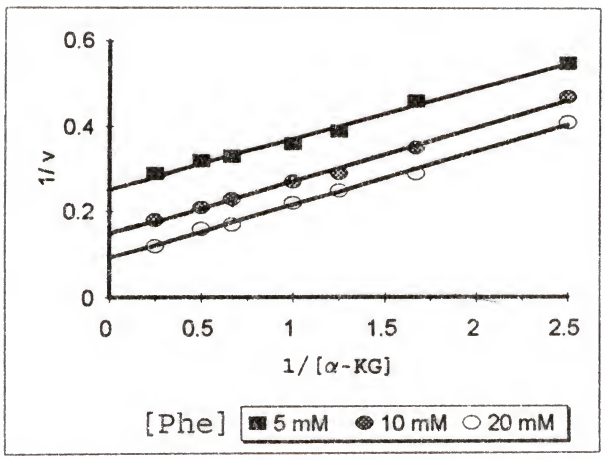


A-2



B

B-1



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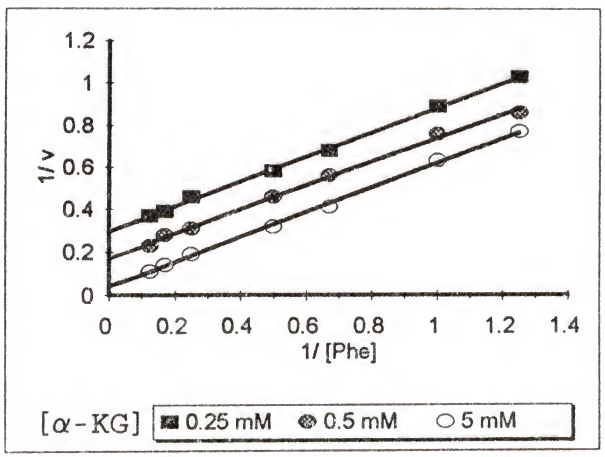
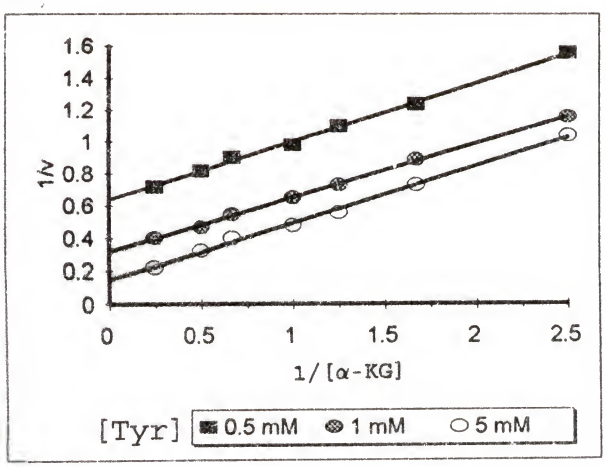


C

C-1



C-2

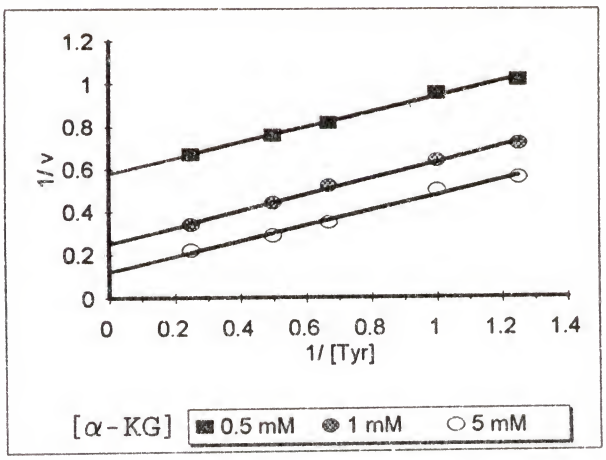
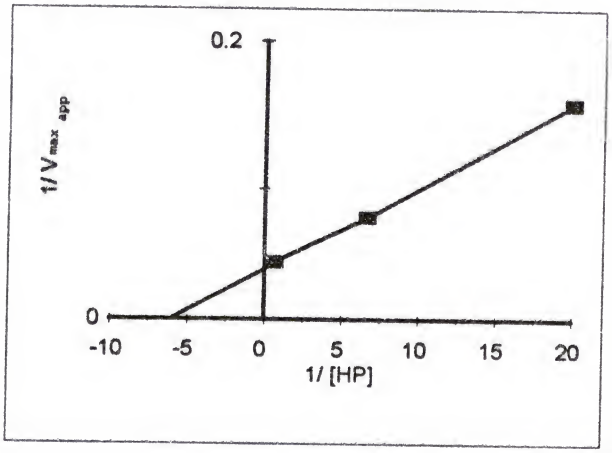


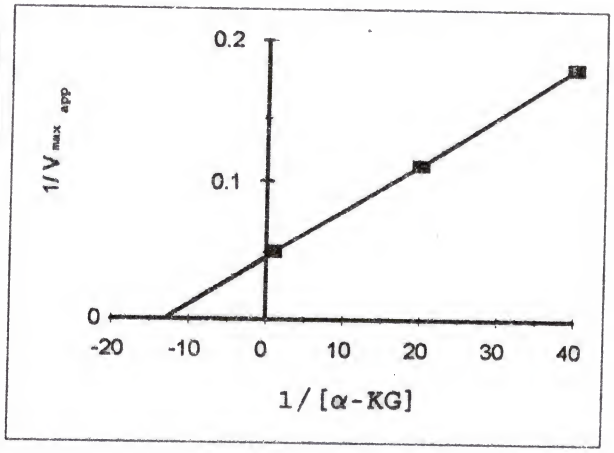
Fig. 10. Replot of 1/v-axis intercept versus various fixed concentrations of the second substrate, based on the data from Fig. 9. A-1 is the replot of A-1 in Fig. 9. The rest of the figures correspond to that in Fig. 9 in the same way.

A

A-1

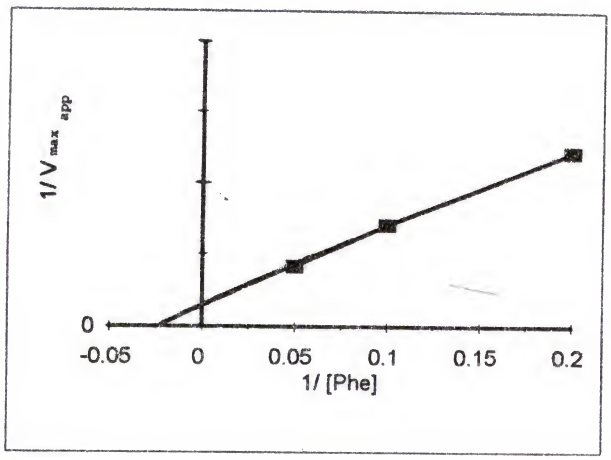


A-2



B

B-1



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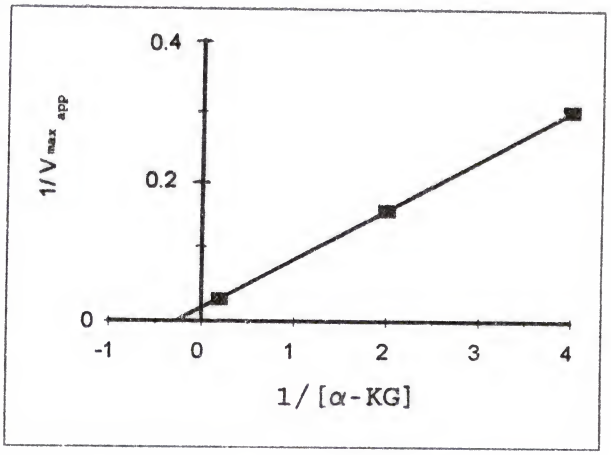
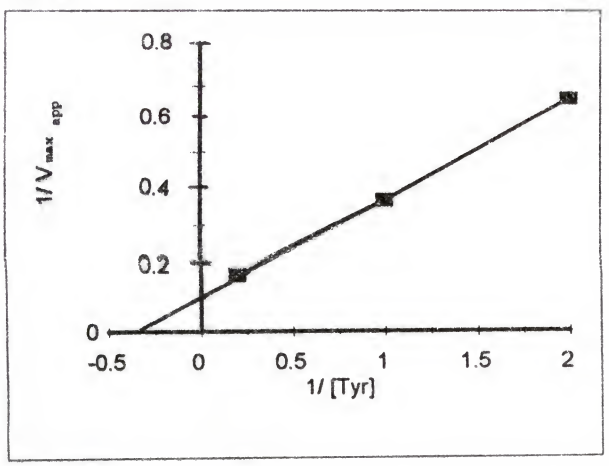


C

C-1



C-2

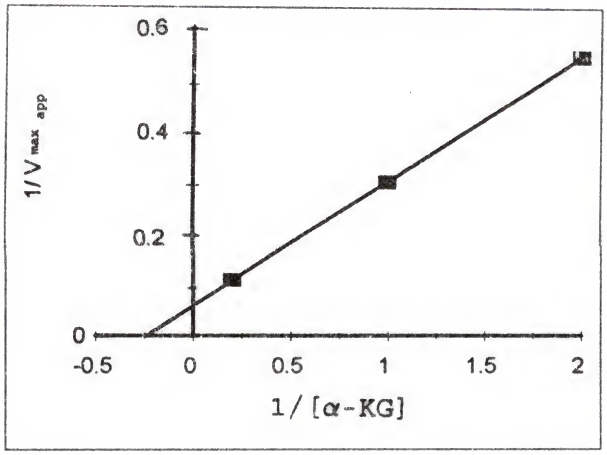


Table 6. Km (mM), V_{max} (nmol/min) values of the enzyme for different substrates. (A) K_m values for different amino donors when α-ketoglutarate was the amino acceptor; (B) K_m values for α-ketoglutarate when different amino donors were used; (C) V_{max} values for three activities.

(A)	Substrate	α-ketoglutarate
K_m	Histidinol phosphate	0.17
	Tyrosine	3.39
	Phenylalanine	43.48

(B)		Histidinol phosphate	Tyrosine	Phenylalanine
K_m	α-Keto-glutarate	0.08	4.08	3.85

(C)	IAT	HAI	PAT
V_{max}	25.52	14.51	46.4

Abbreviations: IAT: imidazole acetol phosphate aminotransferase; HAT: p-hydroxyphenylpyruvate aminotransferase; PAT: phenylpyruvate aminotransferase.

SUMMARY AND CONCLUSION

Identification of the Gene Upstream of tyrC and its Product

The conclusion that the gene upstream of tyrC encodes IAP aminotransferase is strongly supported. The gene complements E. coli hisC mutant UTH780. E. coli transformants possess IAP aminotransferase activity. The calculated protein mass from the deduced amino acid sequence agreed well with the subunit size determined for the purified enzyme by SDS-PAGE. The relative K_M values of the purified enzyme for the alternative substrates showed that histidinol phosphate is by far the favored substrate. The identity of nucleotide sequence of hisH with other known IAP aminotransferase is within a significant range (25%-35%) (Table 7).

The amino acid sequence Ser-X-Y-Lys is a structural feature common to most of the known PLP binding sites (Tanase, et al., 1979). However, Z. mobilis HisH exhibits a replacement of the serine residue with the related threonine residue. The multiple alignment (Fig. 11, Fig. 12) shows that this motif is in fact a distinctive feature of the IAP aminotransferase cluster.

Another stretch of invariant residues (200-203) NPTG can be seen to be highly conserved by inspection of Fig. 11. This motif has been identified as the interdomain interface

Table 7. Homology of microbial IAP aminotransferases.

IAP aminotransferase from:	% identity (with *Zymomonas mobilis* IAP aminotransferase)	% similarity
Bacillus subtilis	35.5%	54.4%
Halobacterium volcanii	31.4%	52.0%
Streptomyces coelicolor	28.6%	51.1%
Saccharomyces cerevisiae	25.8%	49.2%
Escherichia coli	25.6%	46.7%
Salmonella typhimurium	24.6%	50.3%

(Cubellis *et al.*, 1989). It is worth noting that the deduced amino acid sequence reported for *S. typhimurium* from GenBank has a totally unrelated residues (11.8% identity) in this area (184-204), compared with *E. coli* (Fig. 11). Therefore several frame-shift corrections had been tried to see if there might be any evidence of error in gene sequencing. It was found that with a one-base shift in the appropriate area, an amino acid sequence having 82.4% identity with that of *E. coli* was readily obtained which possessed the NPTG motif.

```
S. typhimurium          TAQKWCSFVAPIILPDN
(original)                 |       |
E. coli                 DGVKAVYVCSPNNPTGQ
                        ||  |  |  ||||||||||
S. typhimurium          DGTKVVFVCSPNNPTGQ
(corrected)
```

Fig. 11. Multiple alignment of some aspartate, aromatic,and histidine-pathway aminotransferases. Abbreviations: Ecoaspc, E. coli aspartate aminotransferase; Ecotyrb, E. coli aromatic aminotransferase; Rm-Aroat, Rhizobium meliloti aromatic aminotransferase; Rataspat, Rat aspartate aminotransferase; Zm-Hish, Z. mobilis IAP aminotransferase; Bsuhisha, B. subtilis IAP aminotransferase; Hv-Hish, H. volcanii IAP aminotransferase; Ecohisc, E. coli IAP aminotransferase, St-Hisc, S. typhimurium IAP aminotransferase; Yeast-His5, Saccharomyces cerevisiae IAP aminotransferase; Sc-Hisc, S. coelicolor IAP aminotransferase. The residues marked with asterisk were discussed in the text.

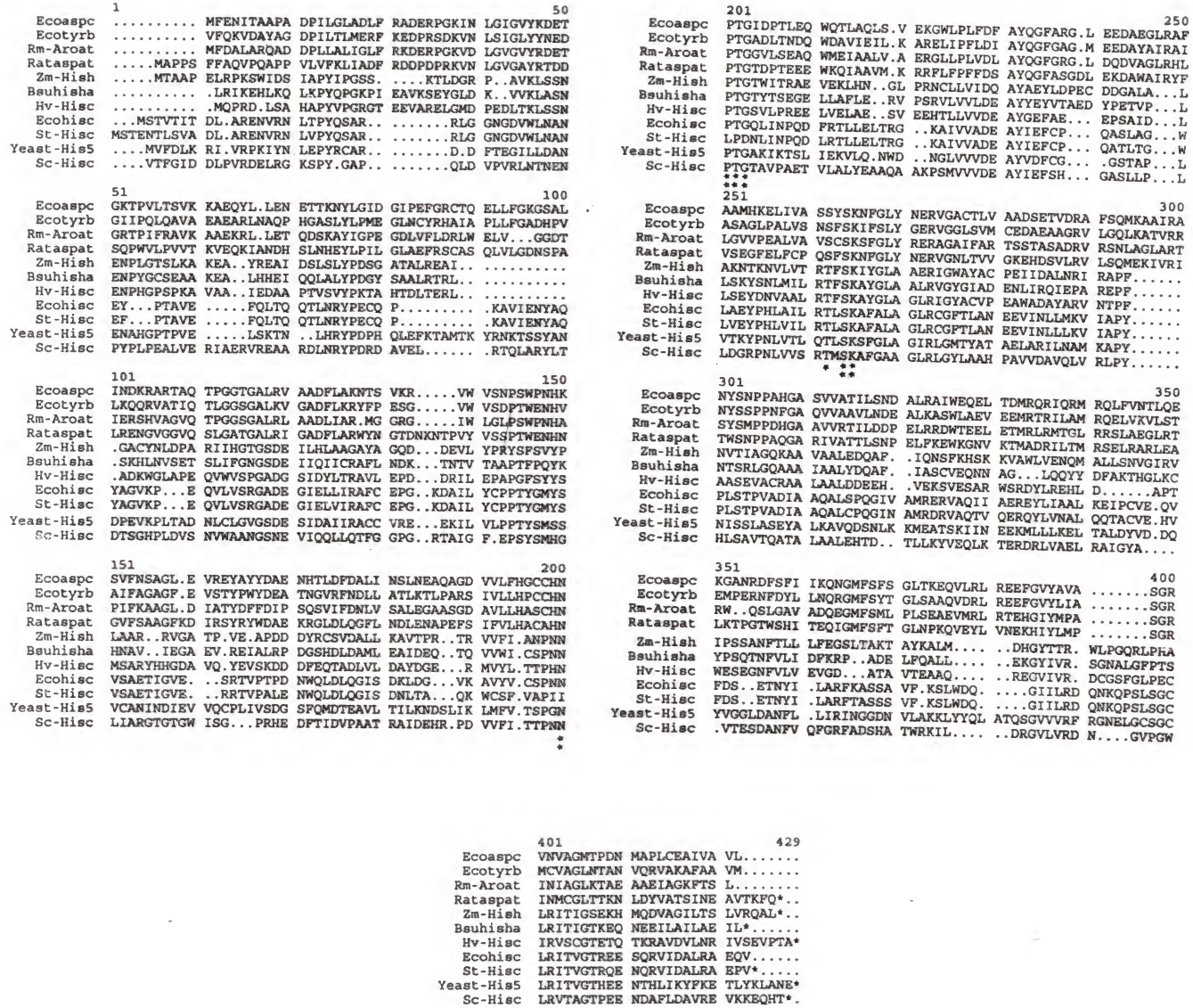
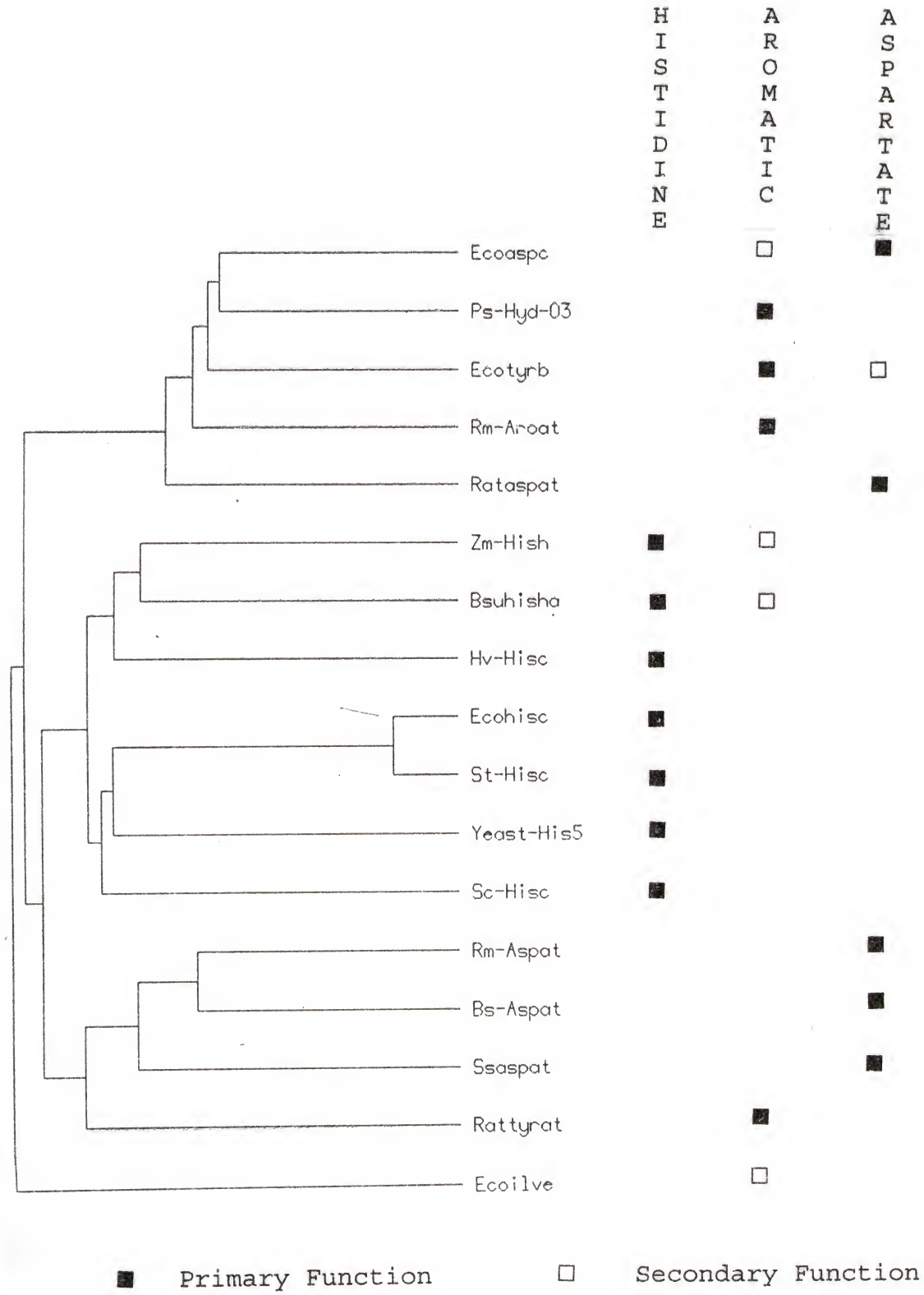

1 50
Ecoaspc ..... MFENITAAPA DPILGLADLF RADERPGKIN LGIGVYKDET
Ecotyrb ..... VFQKVDAYAG DPILTLMERF KEDPRSDKVN LSIGLYYNED
Rm-Aroat ..... MFDALARQAD DPLLALIGLF RKDERPGKVD LGVGVYRDET
Rataspat .....MAPPS FFAQVPQAPP VLVFKLIADF RDDPDPRKVN LGVGAYRTDD
Zm-Hish .....MTAAP ELRPKSWIDS IAPYIPGSS. ....KTLDGR P..AVKLSSN
Bsuhisha ..... .LRIKEHLKQ LKPYQPGKPI EAVKSEYGLD K..VVKLASN
Hv-Hisc ..... .MQPRD.LSA HAPYVPGRGT EEVARELGMD PEDLTKLSSN
Ecohisc ...MSTVTIT DL.ARENVRN LTPYQSAR.. .....RLG GNGDVWLNAN
St-Hisc MSTENTLSVA DL.ARENVRN LVPYQSAR.. .....RLG GNGDVWLNAN
Yeast-His5 ....MVFDLK RI.VRPKIYN LEPYRCAR.. .....D.D FTEGILLDAN
Sc-Hisc ....VTFGID DLPVRDELRG KSPY.GAP.. .....QLD VPVRLNTNEN
51 100
Ecoaspc GKTPVLTSVK KAEQYL.LEN ETTKNYLGID GIPEFGRCTQ ELLFGKGSAL
Ecotyrb GIIPQLQAVA EAEARLNAQP HGASLYLPME GLNCYRHAIA PLLFGADHPV
Rm-Aroat GRTPIFRAVK AAEKRL.LET QDSKAYIGPE GDLVFLDRLW ELV...GGDT
Rataspat SQPWVLPVVT KVEQKIANDH SLNHEYLPIL GLAEFRSCAS QLVLGDNSPA
Zm-Hish ENPLGTSLKA KEA..YREAI DSLSLYPDSG ATALREAI.. ....
Bsuhisha ENPYGCSEAA KEA..LHHEI QQLALYPDGY SAALRTRL.. ....
Hv-Hisc ENPHGPSPKA VAA..IEDAA PTVSVYPKTA HTDLTERL.. ....
Ecohisc EY...PTAVE .....FQLTQ QTLNRYPECQ P......... .KAVIENYAQ
St-Hisc EF...PTAVE .....FQLTQ QTLNRYPECQ P......... .KAVIENYAQ
Yeast-His5 ENAHGPTPVE .....LSKTN ..LHRYPDPH QLEPKTAMTK YRNKTSSYAN
Sc-Hisc PYPLPEALVE RIAERVREAA RDLNRYPDRD AVEL..... .RTQLARYLT
101 150
Ecoaspc INDKRARTAQ TPGGTGALRV AADFLAKNTS VKR.....VW VSNPSWPNHK
Ecotyrb LKQQRVATIQ TLGGSGALKV GADFLKRYFP ESG.....VW VSDPTWENHV
Rm-Aroat IERSHVAGVQ TPGGSGALRL AADLIAR.MG GRG.....IW LGLPSWPNHA
Rataspat LRENGVGGVQ SLGATGALRI GADFLARWYN GTDNKNTPVY VSSPTWENHN
Zm-Hish .GACYNLDPA RIIHGTGSDE ILHLAAGAYA GQD...DEVL YPRYSFSVYP
Bsuhisha .SKHLNVSET SLIPGNGSDE IIQIICRAFL NDK...TNTV TAAPTFPQYK
Hv-Hisc .ADKWGLAPE QVWVSPGADG SIDYLTRAVL EPD...DRIL EPAPGFSYYS
Ecohisc YAGVKP...E QVLVSRGADE GIELLIRAFC EPG..KDAIL YCPPTYGMYS
St-Hisc YAGVKP...E QVLVSRGADE GIELVIRAFC EPG..KDAIL YCPPTYGMYS
Yeast-His5 DPEVKPLTAD NLCLGVGSDE SIDAIIRACC VRE...EKIL VLPPTYSMSS
Sc-Hisc DTSGHPLDVS NVWAANGSNE VIQQLLQTFG GPG..RTAIG F.EPSYSMHG
151 200
Ecoaspc SVFNSAGL.E VREYAYYDAE NHTLDFDALI NSLNEAQAGD VVLFHGCCHN
Ecotyrb AIFAGAGF.E VSTYPWYDEA TNGVRFNDLL ATLKTLPARS IVLLHPCCHN
Rm-Aroat PIFKAAGL.D IATYDFFDIP SQSVIFDNLV SALEGAASGD AVLLHASCHN
Rataspat GVFSAAGFKD IRSYRYWDAE KRGLDLQGFL NDLENAPEFS IFVLHACAHN
Zm-Hish LAAR..RVGA TP.VE.APDD DYRCSVDALL KAVTPR..TR VVFI.ANPNN
Bsuhisha HNAV..IEGA EV.REIALRP DGSHDLDAML EAIDEQ..TQ VVWI.CSPNN
Hv-Hisc MSARYHHGDA VQ.YEVSKDD DFEQTADLVL DAYDGE...R MVYL.TTPHN
Ecohisc VSAETIGVE. ..SRTVPTPD NWQLDLQGIS DKLDG...VK AVYV.CSPNN
St-Hisc VSAETIGVE. ..RRTVPALE NWQLDLQGIS DNLTA...QK WCSF.VAPII
Yeast-His5 VCANINDIEV VQCPLIVSDG SFQMDTEAVL TILKNDSLIK LMFV.TSPGN
Sc-Hisc LIARGTGTGW ISG...PRHE DFTIDVPAAT RAIDEHR.PD VVFI.TTPNN
201 250
Ecoaspc PTGIDPTLEQ WQTLAQLS.V EKGWLPLFDF AYQGFARG.L EEDAEGLRAF
Ecotyrb PTGADLTNDQ WDAVIEIL.K ARELIPFLDI AYQGFGAG.M EEDAYAIRAI
Rm-Aroat PTGGVLSEAQ WMEIAALV.A ERGLLPLVDL AYQGFGRG.L DQDVAGLRHL
Rataspat PTGTDPTEEE WKQIAAVM.K RRFLFPFFDS AYQGFASGDL EKDAWAIRYF
Zm-Hish PTGTWITRAE VEKLHN..GL PRNCLLVIDQ AYAEYLDPEC DDGALA...L
Bsuhisha PTGTYTSEGE LLAFLE..RV PSRVLVVLDE AYYEYVTAED YPETVP...L
Hv-Hisc PTGSVLPREE LVELAE..SV EEHTLLVVDE AYGEFAE... EPSAID...L
Ecohisc PTGQLINPQD FRTLLELTRG ..KAIVVADE AYIEFCP... QASLAG...W
St-Hisc LPDNLINPQD LRTLLELTRG ..KAIVVADE AYIEFCP... QATLTG...W
Yeast-His5 PTGAKIKTSL IEKVLQ.NWD ..NGLVVVDE AYVDFCG... .GSTAP...L
Sc-Hisc PTGTAVPAET VLALYEAAQA AKPSMVVVDE AYIEFSH... GASLLP...L
251 300
Ecoaspc AAMHKELIVA SSYSKNFGLY NERVGACTLV AADSETVDRA FSQMKAAIRA
Ecotyrb ASAGLPALVS NSFSKIFSLY GERVGGLSVM CEDAEAAGRV LGQLKATVRR
Rm-Aroat LGVVPEALVA VSCSKSFGLY RERAGAIFAR TSSTASADRV RSNLAGLART
Rataspat VSEGFELFCP QSFSKNFGLY NERVGNLTVV GKEHDSVLRV LSQMEKIVRI
Zm-Hish AKNTKNVLVT RTFSKIYGLA AERIGWAYAC PEIIDALNRI RAPF.....
Bsuhisha LSKYSNLMIL RTFSKAYGLA ALRVGYGIAD ENLIRQIEPA REPF.....
Hv-Hisc LSEYDNVAAL RTFSKAYGLA GLRIGYACVP EAWADAYARV NTPF.....
Ecohisc LAEYPHLAIL RTLSKAFALA GLRCGFTLAN EEVINLLLKV IAPY.....
St-Hisc LVEYPHLVIL RTLSKAFALA GLRCGFTLAN EEVINLLMKV IAPY.....
Yeast-His5 VTKYPNLVTL QTLSKSFGLA GIRLGMTYAT AELARILNAM KAPY.....
Sc-Hisc LDGRPNLVVS RTMSKAFGAA GLRLGYLAAH PAVVDAVQLV RLPY.....
301 350
Ecoaspc NYSNPPAHGA SVVATILSND ALRAIWEQEL TDMRQRIQRM RQLFVNTLQE
Ecotyrb NYSSPPNFGA QVVAAVLNDE ALKASWLAEV EEMRTRILAM RQELVKVLST
Rm-Aroat SYSMPPDHGA AVVRTILDDP ELRRDWTEEL ETMRLRMTGL RRSLAEGLRT
Rataspat TWSNPPAQGA RIVATTLSNP ELFKEWKGNV KTMADRILTM RSELRARLEA
Zm-Hish NVTIAGQKAA VAALEDQAF. .IQNSFKHSK KVAWLVENQM ALLSNVGIRV
Bsuhisha NTSRLGQAAA IAALYDQAF. .IASCVEQNN AG...LQQYY DFAKTHGLKC
Hv-Hisc AASEVACRAA LAALDDEEH. .VEKSVESAR WSRDYLREHL D......APT
Ecohisc PLSTPVADIA AQALSPQGIV AMRERVAQII AEREYLIAAL KEIPCVE.QV
St-Hisc PLSTPVADIA AQALCPQGIN AMRDRVAQTV QERQYLVNAL QQTACVE.HV
Yeast-His5 NISSLASEYA LKAVQDSNLK KMEATSKIIN EEKMLLLKEL TALDYVD.DQ
Sc-Hisc HLSAVTQATA LAALEHTD.. TLLKYVEQLK TERDRLVAEL RAIGYA....
351 400
Ecoaspc KGANRDFSFI IKQNGMFSFS GLTKEQVLRL REEFGVYAVA ....SGR
Ecotyrb EMPERNFDYL LNQRGMFSYT GLSAAQVDRL REEFGVYLIA ....SGR
Rm-Aroat RW..QSLGAV ADQEGMFSML PLSEAEVMRL RTEHGIYMPA ....SGR
Rataspat LKTPGTWSHI TEQIGMFSFT GLNPKQVEYL VNEKHIYLMP ....SGR
Zm-Hish IPSSANFTLL LFEGSLTAKT AYKALM.... ..DHGYTTR. WLPGQRLPHA
Bsuhisha YPSQTNFVLI DFKRP..ADE LFQALL.... ..EKGYIVR. SGNALGFPTS
Hv-Hisc WESEGNFVLV EVGD...ATA VTEAAQ.... ..REGVIVR. DCGSFGLPEC
Ecohisc FDS..ETNYI .LARFKASSA VF.KSLWDQ. ....GIILRD QNKQPSLSGC
St-Hisc FDS..ETNYI .LARFTASSS VF.KSLWDQ. ....GIILRD QNKQPSLSGC
Yeast-His5 YVGGLDANFL .LIRINGGDN VLAKKLYYQL ATQSGVVVRF RGNELGCSGC
Sc-Hisc .VTESDANFV QPGRFADSHA TWRKIL.... ..DRGVLVRD N....GVPGW
401 429
Ecoaspc VNVAGMTPDN MAPLCEAIVA VL.....
Ecotyrb MCVAGLNTAN VQRVAKAFAA VM.....
Rm-Aroat INIAGLKTAE AAEIAGKFTS L.....
Rataspat INMCGLTTKN LDYVATSINE AVTKFQ*..
Zm-Hish LRITIGSEKH MQDVAGILTS LVRQAL*..
Bsuhisha LRITIGTKEQ NEEILAILAE IL*.....
Hv-Hisc IRVSCGTETQ TKRAVDVLNR IVSEVPTA*
Ecohisc LRITVGTREE SQRVIDALRA EQV.....
St-Hisc LRITVGTRQE NQRVIDALRA EPV*.....
Yeast-His5 LRITVGTHEE NTHLIKYFKE TLYKLANE*
Sc-Hisc LRVTAGTPEE NDAFLDAVRE VKKEQHT*.

Fig. 12. Clustering relationship of three sub-families within a homologous family of aminotransferases from figure of pileup program and their primary and secondary functions. Abbreviations: Ecoaspc, E. coli aspartate aminotransferase; Ps-Hyd-o3, Pseudomonas aeruginosa aromatic aminotransferase; Ecotyrb, E. coli aromatic aminotransferase; Rm-Aroat, Rhizobium meliloti aromatic aminotransferase; Rataspat, Rat aspartate aminotransferase; Zm-Hish, Z. mobilis IAP aminotransferase; Bsuhisha, B. subtilis IAP aminotransferase; Hv-Hish, H. volcanii IAP aminotransferase; Ecohisc, E. coli IAP aminotransferase, St-Hisc, S. typhimurium IAP aminotransferase; Yeast-His5, S. cerevisiae IAP aminotransferase; Sc-Hisc, S. coelicolor IAP aminotransferase; Rm-Aspat, R. meliloti aspartate aminotransferase; Bs-Aspat, Bacillus sp. aspartate aminotransferase; Ssaspat, Sulfolobus solfataricus aspartate aminotransferase; Rattyrat, Rat tyrosine aminotransferase; Ecoilve, E. coli branched-chain amino acid aminotransferase.


HISTIDINE
AROMATIC
ASPARTATE
Ecoaspc
Ps-Hyd-03
Ecotyrb
Rm-Aroat
Rataspat
Zm-Hish
Bsuhisha
Hv-Hisc
Ecohisc
St-Hisc
Yeast-His5
Sc-Hisc
Rm-Aspat
Bs-Aspat
Ssaspat
Rattyrat
Ecoilve
■ Primary Function
□ Secondary Function

Physiological Role of Imidazole Acetol Phosphate Aminotransferase

From genetic and enzymological data, the basic physiological function of hisH is probably directed to histidine biosynthesis. However IAP aminotransferase in Z. mobilis also catalyzes the reactions of phenylpyruvate aminotransferase and p-hydroxyphenylpyruvate aminotransferase. Since hisH is linked to tyrC, it is possible that the aminotransferase function extends to the pathway of aromatic amino acid biosynthesis as well. The different activities coeluted during a purification procedure which yielded a single band following SDS-PAGE whose N-terminal amino acid sequence corresponding to that deduced from the gene sequence. The purified enzyme showed similar physical and enzymological properties, regardless of what substrate combinations were used. These included tolerance to heat, sensitivity to freeze-thaw, and lack of any requirement for added PLP. Thus, it is concluded that IAP aminotransferase and aromatic aminotransferase are synonymous. But, We do not know yet to what extent IAP aminotransferase overlap the function of aromatic aminotransferase in vivo. Is it the basic aromatic aminotransferase in Z. mobilis, a backup enzyme, or neither?

The K_m value for histidinol phosphate is significant smaller than those of aromatic amino-donor, thus the high affinity of the enzyme for histidinol phosphate is consistant with the expectation that the enzyme basic function in histidine pathway. We were expecting the K_m for α-

ketoglutarate were the same regardless what amino-donor was used. However, the K_m for α-ketoglutarate are actually 50 fold higher when histidinol phosphate is the amino-donor than those when aromatic amino acids were the substrates. An explanation has been proposed that the imidazole acetol phosphate produced from histidinol phosphate in the first half step may not be released before the α-ketoglutarate goes on. The imidazole acetol phosphate bound may induce a conformational change of the enzyme which leads a high affinity to the α-ketoglutarate, while phenylpyruvate and p-hydroxyphenylpyruvate bound may not cause such an induction.

Gene Organization Around hisH Gene

The hisH is located upstream of tyrC. The stop codon (TAA) of hisH and the start codon (GTG) of tyrC are adjacent with no intervening bases. Upstream of the hisH is an AT rich region, where an E. coli-like promoter can be found. Within hisH there is another potential internal promoter region which can be recognized by E. coli RNA polymerase and start the transcription of tyrC (Zhao et al., 1993). Downstream of tyrC, 18 bp away from stop codon (TAA), there is a potential RNA polymerase terminator structure consisting of a stem-loop followed by tandem T's: The DNA structure of hisH and tyrC

```
                    TA
      5'-AGCCGTCAGA  T
         ||||||||||                ΔG= -15.9 kcal
3'-TTTTTTCGGCAGTCT  T
                    TT
```

strongly suggest that hisH and tyrC may be organized as an operon.

P: promoter; S: Start of hisH; S': Stop of hisH and start of tyrC; Term: Terminator.

No functionally complemented transformants were obtained when any of the cosmids containing trpFBA or trpC were electroporated into E. coli hisC and tyrA mutants. This indicates that hisH-tyrC was not closely linked with the forgoing tryptophan pathway genes, in contrast to the gene organization shown in B. subtilis (Babitzke et al., 1992). This is not surprising since it was already known that genes involved in tryptophan biosynthesis are separated (Eddy et al., 1988) instead of operonic as in B. subtilis.

Although we have no evidence to show that hisH-tyrC is a member of any larger gene complex or "supraoperon", hisH-tyrC cluster by itself may also play an interesting role in vivo. In E. coli and S. typhimurium, serC (encoding 3-phosphoserine aminotransferase) and aroA (encoding 5-enolpyruvylshikimate 3-phosphate synthase) exist as a mixed function operon (Duncan and Coggins, 1986; Hoiseth and Stocker, 1985), the coordinate expression of which can proceed efficiently in response to iron starvation. It has also been revealed that in E. coli, all the genes cloned encoding the enzymes involving the pyridoxal-5'-phosphate biosynthesis form complex operon with genes whose function are not directly linked to the coenzyme

biosynthesis (Lam and Winkler, 1992; Roa *et al*., 1989).The *hisH*-*tyrC* in our case is probably another example of the growing list of the complex operon. It is possible that the coordinate production of end products or even intermediates in histidine and tyrosine pathway may serve to maintain some physiological balance.

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BIOGRAPHICAL SKETCH

Wei Gu was born on March 20, 1964, in Shanghai, China. She completed her elementary and high school education in Jinan City, Shandong Province. In 1981, she enrolled in the Shandong University, where she majored in microbiology. In 1985, She received her B.S. degree. In the same year, she attended the Shanghai Institute of Plant Physiology, Academia Sinica and worked there until 1988. In 1989, she joined the research group of Dr. Roy A. Jensen where she pursued enzymological and molecular genetic studies of the imidazole acetol phosphate aminotransferase. Under the supervision of Dr. Roy A. Jensen, she received her M.S. degree in August, 1993.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Roy A. Jensen

Roy A. Jensen, Chair
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Lonnie O. Ingram

Lonnie O. Ingram
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Dennis E. Duggan

Dennis E. Duggan
Associate Professor of Microbiology
and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Keelnatham T. Shanmugam

Keelnatham T. Shanmugam
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Charles M. Allen Jr.

Charles M. Allen Jr.
Professor of Biochemistry and
Molecular Biology

This thesis was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

August, 1993

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School